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Human Papillomavirus Detection by Hybrid Capture in Paired Cervicovaginal Lavage and Cervical Biopsy Specimens

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Infection of the uterine cervix with human papillomavirus (HPV) is associated with dysplastic lesions that may progress to malignancy. Certain HPV types are associated with higher risk of cervical cancer than other genital HPVs. The goal of this study was to determine if cells obtained by cervicovaginal lavage contain similar HPV types as paired cervical biopsy in women referred because of abnormal cervical cytology. Thirty-four paired lavage and biopsy samples were analyzed for HPV DNA by hybrid capture, using "low risk" (HPV types 6, 11, and related types and "high risk" group (HPV types 16, 18, and related types) HPV. HPV was detected in 24 lavage samples and 18 biopsies. High risk types were predominant. In 14 of 18 HPV-positive biopsies, the paired lavage was also positive for the same HPV group. Four biopsies were HPV-positive at low levels, and the paired lavage was HPV- negative. The mean viral copy numbers of the biopsies from patients with positive and negative lavage samples were 2.7 and 0.1, respectively ($P = .02$). Ten low level HPV infections were detected by lavage that were not detected by biopsy. HPV detection by hybrid capture in cells obtained by cervicovaginal lavage reflects the results of HPV testing in cervical biopsies. © 1996 Wiley-Liss, Inc.

KEY WORDS: human papillomavirus, diagnosis, hybrid capture, cervicovaginal lavage

INTRODUCTION

Infection of the uterine cervix with human papillomavirus (HPV) is associated with dysplastic lesions that may progress to malignancy [zur Hausen, 1991]. Certain HPV types, such as HPV 16 and HPV 18, are associated with higher risk of cervical cancer than other genital HPVs, such as HPV types 6 and 11. Infection with either "low risk" or "high risk" HPV types is associated with cytologic abnormalities [Kiviat and Koutsky, 1993; Nuovo et al., 1991]. While the Papanicolaou smear (Pap smear) has been effective in reducing the incidence of cervical cancer, there are several problems associated with this method [Cassidy et al., 1988]. Cervical cancer may be found in patients with negative results of cervical cytology [Mitchell et al., 1990]. Another significant problem is determining the appropriate approach to the large number of borderline smears (Atypical Squamous cells of Uncertain Significance, or ASCUS) and mildly dysplastic smears. Cervical biopsies from patients with these low grade cytologic abnormalities show a wide range of pathologic abnormalities [Soutter et al., 1986]. In addition, cervical cytologic testing can miss small lesions that may contain areas of high grade dysplasia [Koss, 1989].

Detection and quantification of human papillomavirus in cervical cells may be useful in making management decisions in women with low grade abnormalities of cervical cytology, and perhaps even in women with normal cytology. Direct analysis of tissue for HPV requires colposcopic- directed biopsy. A simple, noninvasive method to sample cervical cells for HPV detection could be used in epidemiologic studies and in selected cases of patient management. This study was performed to determine if cells obtained by cervicovaginal lavage contain similar HPV types, when grouped according to the relative risk of malignant progression, as those HPVs found in paired cervical biopsies.

While it is now clear that HPV infection is associated with cytologic abnormalities of the cervix, including cervical cancer, there is no consensus regarding the usefulness of HPV detection in making patient management

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decisions [Roman and Fife, 1989]. At present, HPV testing is not routinely performed on cervical cells or tissues. Once cytologic abnormalities are found, patients are generally referred for colposcopy. Biopsy of the cervix, under colposcopic visualization, with subsequent pathologic analysis, provides a histologic diagnosis on which further therapeutic decisions can be based.

Several recent studies suggest a role for HPV detection and quantification in patient management decisions [Bavin et al., 1992, 1993; Cuzick et al., 1992, 1994]. If such a role for HPV detection exists, then a safe, simple method of HPV testing and quantitation would be important if such a method reflected the HPV types actually present in cervical tissue. In this study we tested the hypothesis that DNA purified from cells obtained by cervicovaginal lavage and paired cervical biopsies contain similar HPV types with regard to cancer risk. We carried out repeat cervical cytology followed by cervicovaginal lavage, and cervical biopsy on patients referred for colposcopy because of abnormal cervical cytology. Total cellular DNA was extracted from the paired lavage and biopsy samples from each patient. Hybrid capture was undertaken to determine if HPVs corresponding to "low" or "high risk" types were present, and to determine the amount of HPV in the samples. Hybrid capture is a sensitive and specific assay for HPV in clinical samples [Brown et al., 1993; Farthing et al., 1994]. Good interlaboratory reliability has been established for the hybrid capture assay in detecting HPV [Schiffman et al., 1995]. Quantitative information reflecting the amount of HPV in the infected tissue can be obtained with hybrid capture [Brown et al., 1993].

METHODS

Patient Enrollment and Collection of Samples

Institution Review Board approval was granted for this protocol, and all participants provided informed consent. All patients attending the Regenstrief Colposcopy Clinic were considered eligible if they were 18 years of age or older and were not pregnant. Patients had been referred to the clinic because of abnormal cervical cytology. Some patients were referred because of a single abnormal smear while others had repeated smears diagnosed as ASCUS. Two investigators (MG and KR) collected all samples. Following visual examination of the cervix, an Ayres spatula was used to collect cervical cells for repeat cytologic examination with direct smearing onto a glass slide and fixation. After the cytologic scraping, the cervix was lavaged with 10 ml of sterile normal saline using a syringe and blunt ended canula. The lavage fluid was aspirated immediately and placed into a 15 ml centrifuge tube containing penicillin (25 units per ml) and streptomycin (25 ug per ml). Following application of dilute acetic acid to the cervix, several punch biopsies of abnormal appearing areas were obtained and sent for routine histologic examination. One biopsy sample was placed in a tube containing 1 ml of phosphate buffered saline, pH 7.4, for HPV DNA analysis. The cervicovaginal lavage and biopsy specimens were placed on ice and transported to the laboratory for processing, which generally occurred within 2 hours of collection.

Purification of DNA

Cervical biopsy samples were frozen in liquid nitrogen. DNA was extracted from biopsy samples as previously described [Brown et al., 1993]. Briefly, biopsy samples were frozen with liquid nitrogen, then processed with a Braun mikro-dismembrator II (B. Braun Instruments, Melsungen, Germany). The resulting material was solubilized, treated with proteinase K, and extracted with phenol/chloroform/isoamyl alcohol. DNA was precipitated and quantitated by spectrophotometry. The DNA concentration of each sample was adjusted to 50 ng/μl in a total volume of 200 μl for the hybrid capture assay. The cervicovaginal lavage samples were centrifuged at 4,000g for 15 minutes, and DNA extraction buffer was added to the pellet [Brown et al., 1993]. The remaining steps were carried out as described for the biopsy samples. If less than 10 μg of DNA was obtained from the biopsy or cervicovaginal lavage from an individual patient, then the paired samples were not analyzed by hybrid capture and were excluded from the study.

Hybrid Capture Assay

The presence of HPV DNA was detected by using the hybrid capture assay marketed as ViraType Plus® by Digene Diagnostics, Beltsville, MD, as described previously [Brown et al., 1993]. Briefly, RNA probes of 14 HPV types were allowed to hybridize under high stringency conditions to alkali-denatured specimen DNA. Positive specimens were detected by binding the hybridization reaction to tubes coated with a monoclonal antibody to RNA:DNA hybrids. Bound hybrids were detected by the addition of an alkaline phosphatase-conjugated antibody to RNA:DNA hybrids followed by addition of LumiPhos 520® and reading in an Optocomp I luminometer (MGM Instruments, Hamden, CT). The HPV probes used were divided into two pools whose composition is based on the association of each type with genital tract malignancy. Probe group A contained the "low risk" HPV types 6, 11, 42, 43, and 44 while probe group B contained the "high risk" HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56. Positive controls consisted of 1 pg of HPV 11 DNA (for probe group A) or 1 pg of HPV 16 DNA (for probe group B) diluted in 5 μg of HPV-negative DNA, each run in triplicate with each assay. This amount of control HPV DNA corresponded to 0.05 viral copies per cell in the 5 μg total DNA sample. Patient samples were considered positive if the number of relative light units (RLU) read from the luminometer was greater than the mean of the positive control values. The positive control had to be ≥1.5 times the negative control (HPV-negative DNA) for the test to be considered valid. The HPV copy number was determined by multiplying the sample signal/positive control signal ratio times 0.05, the number of HPV copies represented by the 1 pg positive control DNA.

TABLE I. Detection of HPV by Hybrid Capture in Paired Cerviovaginal Lavage and Cervical Biopsy Specimens

	Any HPV positive	A probe positive	B probe positive	A and B probe positive
Lavage (n = 34)	24	5	19	3
Biopsy (n = 34)	18	2	16	0

TABLE II. Comparison of Lavage and Biopsy in Detecting HPV

		Lavage					
		Any HPV		A probe only		B probe only	
		+	−	+	−	+	−
Biopsy	+	14	4	2	0	12	4
	−	10	40	3	29	7	11

RESULTS

Enrollment of Patients and Collection of Samples

Cervial cytology, cervicovaginal lavage, and colposcopy-directed cervical biopsy were performed on 40 patients by one of two investigators. Two patients underwent examination with sample collections on two occasions. The biopsy samples from six patients and the cervicovaginal lavage samples from two patients did not yield the necessary 10 μg of DNA for hybrid capture, so these patients were excluded from further analysis. Most of the samples that yielded insufficient DNA were obtained early in the study, suggesting that experience in extracting DNA from small samples (especially biopsies) or experience with the sampling techniques was important for optimal DNA yields. Thirty-four paired lavage and biopsy samples, or a total of 68 samples, were included in the analysis.

HPV Detection in Lavage and Biopsy Samples

Overall, 42 of 68 samples were positive for HPV DNA in the hybrid capture assay, including 18 of 34 biopsies and 24 of 34 cervicovaginal lavage samples (Table I). HPV DNA was detected in either the lavage, the biopsy, or both samples in 25 of 34 patients (73.5%). The lavage and biopsy from the remaining nine patients were HPV-negative. "Low risk" HPV types (identified by hybridization with the A probe) alone were detected in seven samples in all: two biopsies and five cervicovaginal lavage samples. "High risk" types (identified by hybridization with the B probe) alone were detected in 16 biopsies and 19 lavage samples. Both low and high risk HPV types were detected in three of lavage samples. No biopsy was positive for both probe groups.

Results of HPV detection from lavage samples and paired cervical biopsies were compared (Table II). For all HPV-positive samples, 14 paired lavage and biopsies were positive for the same probe group. The mean HPV copy number of the 14 biopsies from these women was 2.71 copies per cell (range 0.06–11.98). There were four instances in which a positive biopsy was obtained from a woman whose lavage sample was negative for HPV. The mean copy number of these four biopsies was 0.10 (range 0.05–0.17). The mean copy number for the biopsies with concurring lavage samples (2.71) was significantly different from the mean copy number for biopsies with paired HPV-negative lavage samples ($P = .02$ by two tailed t test). Ten HPV infections, including three low risk and seven high risk types, were detected by lavage that were not detected by biopsy. The mean copy number of these 10 lavage samples from women with HPV-negative biopsies was 0.34 copies per cell.

TABLE III. Histopathology of Biopsies Compared With HPV in Biopsies

Biopsy	HPV+	HPV−
I (n = 11)	3 (3B)	8
II (n = 10)	5 (2A, 3B)	5
III (n = 13)	10 (10B)	3

I, normal histology; II, LGSIL; III, HGSIL, CIS, or adenocarcinoma. A = A probe positive. B = B probe positive.

Cervical Cytology and Biopsy Results

The repeat Pap smears taken at the time of lavage and biopsy revealed a range of cytologic findings including normal cytology (seven samples), ASCUS (nine samples), low grade squamous intraepithelial lesion, or LGSIL (10 samples), high grade squamous intraepithelial lesion, and HGSIL (seven samples), and in one smear, adenocarcinoma. Biopsy results included no pathologic abnormality or inflammatory changes (11 cases), LGSIL (10 cases), HGSIL or carcinoma in situ (CIS) (12 cases), and adenocarcinoma (one case). HPV testing revealed a prevalence of high risk types in those biopsies with HGSIL (Table III).

Eleven biopsies were normal or revealed only inflammatory changes; three of these contained high risk HPV DNA and the other eight contained no detectable HPV DNA. Lavage samples from the two of the three patients with histologically normal, HPV-positive biopsies contained HPV DNA of the sam risk group as was detected in the biopsy.

Thirteen biopsies revealed HGSIL (11 cases), carcinoma in situ (one case), or adenocarcinoma (one case). Ten of these 13 biopsies were B probe- positive, indicating the presence of a high risk HPV type. None of these biopsies were positive with the A-probe. Three biopsies revealing HGSIL were HPV-negative, but the lavage samples from two of these three patients were B probe-positive. The biopsy containing invasive adenocarcinoma was positive for high risk HPV DNA with a copy number of 0.07 viral copies per cell. The remaining 10 biopsies were all LGSIL. Five of these biopsies contained HPV DNA, including three with high risk HPV and two with low risk HPV.

Because other studies (involving a larger number of women) have noted an association of high grade dysplasia with higher amounts of HPV 16, we compared the viral copy number in B probe-positive samples with nor-

mal and abnormal histologic findings. The mean viral copy numbers for high risk HPV types detected in normal, LGSIL, and HGSIL biopsies did not differ significantly, but there were only three B probe-positive biopsies in each of the normal and LGSIL groups.

DISCUSSION

It is clear that women with high risk HPV infection of the cervix are at risk for development of high grade dysplasia in a relatively short period of time. Koutsky et al. [1992] prospectively followed 241 women with negative cervical cytologic examinations for up to 4 years. Women were regularly examined and tested for HPV infection by dot-filter hybridization performed on DNA from cervical swabs. The cumulative incidence of cervical intraepithelial neoplasia was 28% among women with a positive test for HPV and 3% among women without detectable HPV DNA. The risk was highest among women infected with HPV type 16 or 18. CIN 2 or 3 developed in 39% of women infected with HPV types 16 or 18 within 24 months. The authors did not address the issue of the quantity of HPV. A prospective study of patients with normal cervical cytology and very low level HPV infection (especially with high risk HPV types) needs to be performed. Such a study could provide a more defined role for the need, or lack of need for HPV testing and typing.

In our study, DNA extracted from cells obtained by cervicovaginal lavage produced positive signals with the same probe group as the biopsy in most cases, suggesting that lavage is a useful procedure to detect cervical HPV infection. There were 10 cases in which the lavage was positive and the biopsy negative. These were low copy number samples, with a mean of 0.34 viral copies per cell. Possible explanations for this observation include very low level infection in the paired cervical biopsy from these 10 patients, below the detection limit of the hybrid capture assay (0.05 copies per cell), or infection of the vaginal mucosa with HPV. In addition, if a normal, uninfected area of the cervix was sampled by biopsy, rather than the infected area, the lavage could be positive when the biopsy received in the laboratory was negative for HPV.

The biopsy was positive for HPV in four cases in which the lavage fluid was negative. These cases are disturbing, because it may be essential that all high risk cervical infections are detected. This finding could mean that HPV testing by hybrid capture on lavage samples lacks adequate sensitivity. In these four cases the mean copy number was 0.1 copies per cell, compared to the mean copy number of 2.7 in biopsies with a paired lavage sample that were HPV-positive. A likely explanation for the negative lavage in these four cases is the dilutional effect of the lavage procedure. The biopsy obviously represents tissue likely to contain relatively high amounts of virus per cell. The lavage, in contrast, samples the entire cervix, including the lesion and adjacent noninfected tissue, and the vaginal mucosa. It is not surprising, therefore, that low level infection of the cervix may produce a negative test in some lavage samples. As mentioned above, the clinical significance of very low level HPV infection is not known.

The viral copy number represents the "burden" of HPV in the infected tissue. Although not the main goal of the study, we compared the mean viral copy number of high risk HPV in normal biopsies, or biopsies classified as LGSIL or HGSIL. No significant differences were found, but our sample sizes in each group were insufficient to detect small differences. Other studies suggest that determining the amount of HPV DNA in a sample may have a role in clinical decision making [Bavin et al., 1992, 1993; Cuzick 1992, 1994]. Cuzick et al. showed that higher levels of HPV 16, determined by PCR, were associated with high grade lesions of the cervix. Although the authors did not find HPV 16 in all cervical smears from women with high grade cervical dysplasia found by cervical biopsy, almost 90% of patients with intermediate or high amounts of HPV 16 DNA had an underlying high grade lesion [Cuzick et al., 1992]. A second study by these authors showed that other "high risk" HPV types in cervical smears were also predictive of high grade lesions in cervical biopsies [Cuzick et al., 1994]. Bavin et al. also showed that higher amounts of HPV 16 DNA in cervical scrapes were associated with high grade dysplasia found on cervical biopsy [Bavin et al., 1993]. These studies should be viewed as using semiquantitative methods. A distinct advantage of the hybrid capture assay is the ability to provide reproducible quantitative data with regards to the amount of HPV in lavage and biopsy samples.

HPV detection and quantification in cervical cells may be useful for clinical decision making. We found a good, but not excellent correlation between the HPV types, when grouped into low or high risk categories, in cervical biopsy and cells collected by lavage from samples containing greater than approximately 0.3 viral copies per cell. Low level infection of the cervix may not be detected in paired lavage samples. Cervicovaginal lavage is a simple, inexpensive procedure for obtaining cellular material for HPV diagnosis. The hybrid capture assay can detect and quantitate HPV DNA in samples obtained by lavage. While knowledge of the specific HPV type could be useful for epidemiologic studies, the method of grouping high and low risk probes greatly simplifies HPV diagnosis.

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